

Kongeriget Danmark

Patent application No.: PA 2003 00443

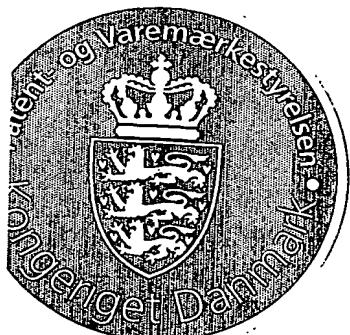
Date of filing: 21 March 2003

Applicant:
(Name and address)
UpFront Chromatography A/S
Lersø Parkallé 42
DK-2100 København Ø
Denmark

Title: Method for high throughput volumes in the fractionation of biomolecules by chromatographic systems

IPC: -

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.



Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

05 April 2004
A handwritten signature in black ink, appearing to read "Pia Petersen".

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

Modtaget PVS

1

21 MRS. 2003

METHOD FOR HIGH THROUGHPUT VOLUMES IN THE FRACTIONATION OF BIO-MOLECULES BY CHROMATOGRAPHIC SYSTEMS

FIELD OF INVENTION

The invention relates to an industrial scale chromatographic process for fractionation and isolation of bio-molecules from fluids, e.g. proteins from milk and whey in a cost-effective manner. The process allows for processing large volumes of fluid in a short time and for improved adsorbent efficiency by means of operating the process at high temperature and high flow rate.

10 BACKGROUND OF THE INVENTION

Generally, a very broad range of different chromatographic processes for industrial scale fractionation and/or isolation of biological molecules such as proteins, lipids, saccharides, lipo-proteins, cells and cells constituents are available.

15 When utilising chromatographic processes for industrial scale production, the production efficiency and economically consequences is a matter of strong considerations. Many attempts have been made in order to improve the efficiency of chromatographic processes, for instance by providing adsorbent particles of smaller sizes, increasing the surface of the adsorbent particle so as to improve the adsorptive capacity of the adsorbent towards a bio-molecule.

However, there is still a need for improving the efficiency of chromatographic processes for industrial scale production. In particularly higher productivity may be requested upon isolating or fractionating bio-molecules from fluids with lower content of bio-molecules. For 25 example, the concentration of lactoferrin in bovine skimmed milk is usually small, typically between 80-200 mg/l depending on e.g. the pasteurisation and other pre-treatment history of the skimmed milk.

Thus, a process allowing for higher productivity is of particular interest in fractionation and 30 isolation of lactoferrin from milk or whey. WO 02/096215 relates to a method for fractionating lactoferrin from milk or whey using flow rates about 200 to 900 cm/hr. Furthermore, fractionation of immunoglobulins is of interest. WO 98/08603 relates to a method for isolation of immunoglobulins. Conventionally, these methodologies have been carried out using temperatures in the range of about 10°C.

35

The present inventors report herein a method for significant improvement of the productivity of chromatographic processes of industrial scale by providing means for operating the chromatographic processes with very high flow rates and by providing means for improving the adsorbent capacity of an adsorbent. Thus, such processes may allow for
5 more cost effective fractionation and/or isolation of bio-molecules of interest.

Patent 5,596,082 discloses an industrial process for isolation of lactoperoxidase and lactoferrin from milk and milk products with packed bed chromatography using a strong cation exchanger (SP sepharose Big Beads from Amersham). The chromatographic beads
10 described for the process have a mean particle size in the range of 100- 300 microns and working flow rates in the range of 2000 - 3000 cm/hr may be used.

SUMMARY OF INVENTION

The present invention relates to a chromatographic process capable of processing very
15 large volumes of bio-molecule containing fluids in a short time and capable of providing high productivity, while still achieving high purity of the biological molecule isolated by the process. This may be achieved by operating the chromatographic processes at high flow rates and at high temperatures.
20 Thus, in a primary aspect the Invention relates to a general process for fractionating and/or isolation of one or more bio-molecule(s) from a bio-molecule containing fluid comprising the steps of:
25 a) optionally adjusting the pH of the bio-molecule containing fluid;
 b) optionally bringing the bio-molecule containing fluid to a temperature of between 45°C to 80°C;
30 c) applying a volume of said bio-molecule containing fluid to a chromatographic column comprising an adsorbent, said chromatographic column is operated with a temperature of between 45°C to 80°C and a linear flow rate of at least 1.500 cm/hour;
 d) optionally washing the column;
35 e) eluting at least one bio-molecule from the adsorbent.

One object of the present Invention is to provide an improved process for industrial-scale fractionation and/or Isolation of proteins such as lactoferrin from suitable body fluids or fluids derived therefrom including milk and whey, using either packed bed chromatography or EBA chromatography.

5

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a SDS-PAGE illustrating the binding and elution profiles of whey proteins isolated with FasLine PRO adsorbent in an EBA process. Lane 1 illustrates the raw sweet whey, lane 2 illustrates flow through fraction 1 from the column, lane 3 illustrates flow

- 10 through fraction 2 from the column, lane 4 illustrates flow fraction 3 from the column, lane 5 illustrates the wash with demineralised water, lane 6 illustrates eluate 1 at pH 5.3, lane 7 illustrates eluate 2 with 20 mM NaOH.

:

DETAILED DESCRIPTION OF THE INVENTION

- 15 The present investigators provide herein evidence that the temperature used during load of bio-molecule containing fluids onto a chromatographic column significantly improves the adsorptive capacity of the adsorbent. As can be derived from example 1, operating a chromatographic process at a temperature of 50°C instead of the conventional 10°C results in doubling of the adsorbent capacity of the adsorbent, i.e. the amount (g) of Lactoferrin
20 adsorbed to 1 l of adsorbent was doubled. Furthermore, upon operating the chromatographic process at 50°C and with flow rates higher than conventional ones (from 1.500 cm/hr to 3.000 cm/hr), the volume of the bio-molecule containing fluid that can be loaded onto the column increases significantly, while still achieving the same high adsorbent capacity (example 2). Thus, upon increasing the linear flow rate during loading
25 of the bio-molecule containing fluid onto the column, the productivity increases. Productivity might be regarded as the amount of bio-molecule that can be adsorbed to 1 litre of adsorbent in 1 hour. As can be seen from example 3, the process time is dramatically reduced upon operating the chromatographic process at higher temperatures, such as 50° in combination with higher linear flow rate, such as 2.100 cm/hr.

30

Thus, the combination of high temperatures and high flow rates seems to be a surprisingly promising approach in increasing the productivity of chromatographic systems, in particular systems of industrial scale where any reduction in costs may be of great commercial importance.

35

Accordingly, in a primary aspect the invention relates to a process for fractionating and/or isolation of one or more bio-molecule(s) from a bio-molecule containing fluid comprising the steps of:

- 5 a) optionally adjusting the pH of the bio-molecule containing fluid;
- b) optionally bringing the bio-molecule containing fluid to a temperature of between 45°C to 80°C;
- 10 c) applying a volume of said bio-molecule containing fluid to a chromatographic column comprising an adsorbent, said chromatographic column is operated with a temperature of between 45°C to 80°C and a linear flow rate of at least 1.500 cm/hour;
- 15 d) optionally washing the column;
- e) eluting at least one bio-molecule from the adsorbent.

Bio-molecules

- 20 As defined herein the term "bio-molecule" is intended to mean any molecule and entity that is obtainable from biological origin having a molecular weight of at least 1000 Daltons. As is to be understood, the bio-molecule may be obtained by use of synthetically means, gene technology and/or fermentation. Furthermore, the bio-molecule may be different to that of the biological origin because of derivatisation of the bio-molecule. Thus, the term
- 25 "bio-molecule" may encompasses bio-molecules that are obtainable from biological origin and derivatives thereof. Typically, such bio-molecules are peptides, proteins, lipids, lipoproteins, polysaccharides or mixtures thereof. Furthermore, in some embodiments of the invention the term "bio-molecule" also encompasses entities obtainable from biological origin having a molecular weight of at least 20,000 D, e. g. DNA (plamide DNA,
- 30 chromosomal DNA, virus DNA), RNA such as virus RNA, or viruses them cells, even bacteria. The term "bio-molecule" is also meant to include cell constituents and cells.

It is contemplated that the process acquires practical importance for bio-molecules of higher molecular weight. Thus, in some embodiments of the invention, the one or more
35 bio-molecule(s) has/have a molecular weight of at least 1500 Daltons, more preferably of at least 2000 Daltons.

As may be understood, the process of the Invention may be applicable for a broad variety of bio-molecules as long as any adsorbent capable of binding the bio-molecule of interest

is available. Therefore, in embodiments of the invention, the one or more bio-molecule(s) is/are selected from the group consisting of peptides, proteins, lipids, lipoproteins, polysaccharides, cell constituents, cells or combinations thereof.

- 5 In interesting embodiments thereof, the one or more bio-molecules is/are selected from:
- Proteins such as lactoferrin, immunoglobulins, β -lactoglobulin, α -lactalbumin, lactoperoxidase, patatein and other proteins from potatoes, enzymes such lysozyme.
 - Lipids such as phospholipids from milk.
 - Polysaccharides such as starches, e.g maize and potato starch, and pectins such as chitosans.

Bio-molecule containing fluid

The process according to the present invention is targeted, at least in part, for industrial or large-scale fractionation processes where large volumes must be handled. Of interest are

- 15 fluids containing bio-molecules in low content, such as fluids that otherwise may be discharged, e.g. process water containing interesting bio-molecules, but in too low content in order to attract any commercial interest. However, bio-molecule containing fluids containing high amounts of bio-molecules are not anticipated by the present invention, and may constitute further interesting embodiments.

- 20 In the present context of the invention, the term "bio-molecule containing fluid" is intended to denote a fluid of biological origin or derived therefrom, which comprises at least one or more bio-molecule within the context of this invention to be fractionated, partially or wholly purified or isolated on an industrial or large scale. Typically, such fluids
- 25 include body fluids or fluids derived therefrom including milk, skimmed milk, whey or other milk derived fluids; blood or fluids derived therefrom; plasma or fluids derived therefrom; serum or fluids derived therefrom; lymph or fluids derived therefrom; urine or fluids derived therefrom; egg white or fluids derived therefrom; or egg yolk or fluids derived therefrom. Also typically, the bio-molecule containing fluid is denoted to include
- 30 fermentation fluids; waste water; process water; plant extracts such as fruit derived fluids; tissue extracts such as fish derived fluids; synthesis mixtures; and fluids derived therefrom.

- Accordingly, in embodiments of the invention the bio-molecule containing fluid is selected
35 from the group consisting of body fluids, fermentation fluids, waste water, process water, plant extracts, tissue extracts, synthesis mixtures and fluids derived therefrom.

In some embodiments of the invention, the bio-molecule containing fluid is process water from the food and/or feed industry, e.g. process water from the production of starches, e.g.

potato starch and/or maize starch. In still other embodiments, the bio-molecule containing fluid is waste water comprising undesirable organic molecules, such as toxins, allergens, pesticides in that the waste water need to be purified from the containment of such undesirable organic molecules before being released to the environment or used for preparation of drinking water.

In presently interesting embodiments, the bio-molecule containing fluid is selected from the group comprising of milk, skimmed milk, whey or any other milk derived fluids.

10 pH adjustment

As may be understood, the bio-molecule containing fluid may before being loaded to a chromatographic column need an adjustment in pH depending on the protein of interest, the ligand chemistry, and the type of bio-molecule containing fluid.

15 In some embodiments of the present invention the bio-molecule containing fluid is pH adjusted prior to being applied to the adsorbent column to facilitate the capture of bio-molecule such as a protein by the adsorbent. This pH may be adjusted to a pH value selected in the entire pH range, preferably from pH 2-13, more preferably from pH 3-11.

20 Chromatographic column

The chromatographic column to be used may be any kind suitable for either EBA (Expanded Bed Adsorption) or suitable for packed bed adsorption or a combination thereof. The chromatographic column may be used in either a batch system or in a continuous system. Thus, in some embodiments of the invention, the chromatographic column is an expanded bed adsorption column and in still other embodiments, the chromatographic column is a packed bed adsorption column.

30 In the present context the term "chromatographic column" relates to any kind of container which can be supplied with at least one inlet and at least one outlet for the application of the bio-molecule containing fluid to the column and subsequent elution of one or more bio-molecule of interest.

35 The fact that the EBA technology generally can work efficiently with non-clarified fluids makes it attractive to implement for the isolation and fractionation of bio-molecules from fluids such as milk, whey fermentation fluids and process water. Compared to packed bed adsorption techniques EBA may offer a robust process comprising fewer steps and thus result in increased yields and an improved process economy. Due to the expansion of the adsorbent bed during execution of an EBA process, EBA columns may further be scaled up to industrial scale without any significant considerations regarding increased back

pressures or breakdown of the process due to clogging of the system which often is a problem when using packed bed columns.

- However, the present state of art within the EBA technology does not adequately address
5 the solution of how to process high volumes of fluids, while still achieving high productivity.

General Expansion Bed Adsorption technology is known to the person skilled in the art and the process of the present invention may be adapted to the processes described in, for
10 example, WO 92/00799, WO 92/18237, WO 97/17132, WO 98/33572, WO 98/08603, WO 00/57982, WO 01/58924, and WO 02/096215.

As may be understood, the process may be specific applicable to industrial scale systems. Thus, in interesting embodiments of the invention, the chromatographic column is a large-
15 scale chromatographic column comprising at least 10 l of sedimented adsorbent, as may be determined as the amount (litre) of adsorbent settled when operating the column without flow. In still interesting embodiments thereof, the chromatographic column is a large-scale chromatographic column comprising from about 50 to 100 l of sedimented adsorbent. Preferably, the amount of sedimented adsorbent is from about 100 to 1000 l,
20 more preferably from about 200 to 900 l, most preferably from about 300 to 800 l.

Furthermore, for industrial scale production, the chromatographic column has a diameter of at least 10 cm, preferably of at least 20 cm, more preferably in the range of from about 50 cm to 200 cm, such as 100 to 150 cm.

25

Temperature

As mentioned conventional methodologies within the field of fractionating and isolating bio-molecules often uses temperatures about room temperature or lower, such as 10°C. However, one objection of the present invention is to apply higher temperatures in
30 chromatographic processes intended for isolation of bio-molecules, although high temperatures may inadversely affect some temperature sensitive bio-molecules. For instance enzymes that may loose their enzymatic capacity upon being exposed to high temperatures, such as above 40°C.

35 The temperature of the bio-molecule containing fluid may be important for reasons of viscosity in that higher temperatures reduces the viscosity, which in turn means that it is easier to run substances through a packed bed column because of less pressure in the column. Running an EBA process at high temperature results in a less expanded bed

meaning that high flow rates can be applied without loosing the adsorbent media in the column effluent.

- In currently interesting embodiments of the invention, the chromatographic column is
- 5 operated at temperatures of at least 45 °C. However, a upper limit may exist, such as 80°C in that even bio-molecules with tolerance for high temperatures may not resist temperatures above 80°C. It is to be understood, that the use of higher temperatures such as 85°C, 90°C, or 95°C are not anticipated.

- 10 As to be understood, in some embodiments of the invention, the bio-molecule containing fluid is equilibrated to the desired temperature before being loaded to the column. Thus, the process of the invention may include the step of bringing the bio-molecule containing fluid to a temperature of between 45°C to 80°C, such as of between 50° and 70°C. However, higher temperatures are not anticipated such as 85°C, 90°C, or 95°C.

15

In current interesting embodiments of the invention, the temperature is between 50 and 70 °C.

Flow rate

- 20 One major advantage of the invention relates to the applicability of higher flow rates than conventional ones, that may account to about 200 cm/hr. According to the present invention, linear flow rates of from about 1.500 to 12.000 cm/hr may be applicable during loading of the bio-molecule containing fluid to the chromatographic column. Preferably, the linear flow rate may be operated within 1.800 to 10.000 cm/hr, such as within 2.000 to
- 25 10.000 cm/hr, such as within linear flow rates of about 3000 cm/hr.

- The utilisation of higher flow rates allows for loading higher volumes of bio-molecule containing fluids within a shorter time than conventionally possible. However, this may depend on the size of column adapted. In current suitable embodiments of the invention,
- 30 the volume to be applied onto the column is from about 2-3500 l/min.

- In other terms, the efficiency of the process as defined herein may be expressed by the volume of bio-containing fluids that can be applied to 1 litre of adsorbent per hour. Thus, in some embodiments of the invention, the volume applied per litre of adsorbent in one
- 35 hour is at least 50 l, preferably at least 100 l, more preferably at least 150 l/min such as at least 200 l/min.

However, in packed bed methodology, higher flow rates may lead to high pressures within the chromatographic column, thus affecting the performance of the chromatographic system

- Including problems with leak and breakdown of equipment. The present investigators have found that the present process, which operates at higher temperatures such as above 45°, allows for operating the chromatographic column with a pressure, as measured over the entire chromatographic column, of at most 10 bar, such as of at most 9, 8, 7, 6 or 5 bar, 5 preferably of at most 4 bar, most preferably of at most 3 bar such as of at most 2.5 bar.

Adsorbent

- In the present context the term "adsorbent" relates to the entire bed present in the chromatographic column and the term "adsorbent particle" are used interchangeably with 10 the term "particle" and relates to the individual single particles which makes up the adsorbent.

- Generally, the term "adsorbent" is meant to characterize any suitable adsorbent used in chromatographic processes such as adsorbents suitable for ion-exchange 15 chromatography, protein A and Protein G affinity chromatography, other affinity chromatography, hydrophobic chromatography, reverse phase chromatography, thiophilic adsorption chromatography and mixed mode adsorption chromatography and the like.
- 20 The flow rate, the size of the particles and the density of the particles all have influence on the expansion of the fluid bed and it is important to control the degree of expansion in such a way to keep the particles inside the column. The degree of expansion may be determined as H/H_0 , where H_0 is the height of the bed in packed bed mode and H is the height of the bed in expanded mode. In a preferred embodiment of the present invention 25 the degree of expansion H/H_0 is in the range of 1.0-20, such as 1.0-10, e.g. 1.0-6, such as 1.2-5, e.g. 1.5-4 such as 4-6, such as 3-5, e.g. 3-4 such as 4-6. In an other preferred embodiment of the present invention the degree of expansion H/H_0 is at least 1.0, such as at least 1.5, e.g. at least 2, such as at least 2.5, e.g. at least 3, such as at least 3.5, e.g. at least 4, such as at least 4.5, e.g. at least 5, such as at least 5.5, e.g. at least 6, such as 30 at least 10, e.g. at least 20.

- The particles size analysis performed and referred to throughout the description and the examples is based on an computerised image analysis of the bead population giving the number of particles at any given particle diameter in relation to the total number of 35 particles analysed in the specific measurement. Typically the total number of particles analysed will be in the range of 250-500 particles). These particle size data may be transferred into the volume percent represented by each particle size by a routine

10

mathematical transformation of the data, calculating the volume of each bead and relating this to the total volume occupied by all beads counted in the measurement.

The particle size distribution according to the invention is preferably defined so that more than 90% of the particles are found between 20-500% of the mean particle diameter, more preferable between 50-200% of the mean particle diameter, most preferable between 50-150% of the mean particle diameter.

Traditionally packed bed materials have a mean diameter less than 100 microns, which enables an efficient binding of the protein. Their disadvantage is their high flow resistance. It is not possible to apply flow rates higher than 500 cm/hr, which is not a problem in analytical applications but for large scale processing it becomes a limiting factor. At flow rates higher than 500 cm/hr the pressure drop over the column material will increase and the bed height will be the limiting factor. If large amounts of substances are to be processed the diameter of the column should be rather large. This requires construction of columns of high standard in order to meet the required adequate distribution of the substances and withstand the high pressures. The cost of such column has a great impact on the process economy.

Also the level of clarification of the feed stream also affects the pressure drop. Traditional packed beds work as depth filters that can clog, resulting in increased pressure drop unless the feed is thoroughly clarified.

In the event where the chromatographic column is an EBA column, the density of the EBA adsorbent particle is found to be highly significant for the applicable flow rates in relation to the maximal degree of expansion of the adsorbent bed possible inside a typical EBA column (e.g. H/H₀ max 3-5) and must be at least 1.3 g/mL, more preferably at least 1.5 g/mL, still more preferably at least 1.8 g/mL, even more preferably at least 2.0 g/mL, most preferably at least 2.3 g/mL in order to enable a high productivity of the process.

As stated, the process of the invention may be operated with use of high flow rates, while still achieving high productivity and efficient adsorption of bio-molecules to the adsorbent. This may at least in part be due to limiting the mean particle diameter of the adsorbent particle. In a preferred embodiment of the present invention the adsorbent particle has a mean particle size of at most 200µm, such as at most 150 µm, particularly at most 120 µm, more particularly at most 100 µm, even more particularly at most 90 µm, even more particularly at most 80 µm, even more particularly at most 70 µm. Typically the adsorbent particle has a mean particle size in the range of 40-150 µm, such as 40-120 µm, e.g. 40-100, such as 40-75, e.g. 40-50 µm. For packed bed methodology, the adsorbent particle

11

size may however not be in the lower range, but such that the adsorbent particle has a mean particle size of at most 200 μm , such as at most 150 μm , particularly at most 120 μm , more particularly at most 100 μm , even more particularly at most 90 μm

- 5 Alternatively expressed, in suitable embodiments of the invention the adsorbent is made of particles, wherein 50% of the number of particles has a particle size of at most 200 μm , particularly at most 175, 150, 120, 100, 90, 80 or at most 70 μm .

The particle size as referred to herein relates to the longest distance as can be measured
10 on the particle.

In a combination of preferred embodiments, where the average particle diameter is 120 μm or less, the particle density is at least 1.6 g/mL, more preferably at least 1.9 g/mL. When the average particle diameter is less than 90 μm the density must be at least 1.8
15 g/mL or more preferable at least 2.0 g/mL. When the average particle diameter is less than 75 μm the density must be at least 2.0 g/mL, more preferable at least 2.3 g/mL and most preferable at least 2.5 g/mL.

The high density of the adsorbent particle is, to a great extent, achieved by inclusion of a
20 certain proportion of a dense non-porous core materials, preferably having a density of at least 4.0 g/mL, such as at least 5.0. Typically, the non-porous core material has a density in the range of about 4.0-25 g/ml, such as about 4.0-20 g/ml, e.g. about 4.0-15 g/mL, such as 12-19 g/ml, e.g. 14-18 g/ml, such as about 6.0-15.0 g/mL, e.g. about 6.0-10 g/ml.
25

The high density of the adsorbent particle is, to a great extent, achieved by inclusion of a certain proportion of a dense non-porous core materials, preferably having a density of at least 4.0 g/mL, such as at least 5.0. Typically, the non-porous core material has a density in the range of about 4.0-25 g/ml, such as about 4.0-20 g/ml, e.g. about 4.0-15 g/mL,
30 such as 12-19 g/ml, e.g. 14-18 g/ml, such as about 6.0-15.0 g/mL, e.g. about 6.0-10 g/ml.

Subsequently, the bio-molecular containing fluid is loaded and the bio-molecules of interest are adsorbed. Particulate material and soluble impurities are optionally removed
35 from the column during the wash.

In a preferred embodiment of the present invention the adsorbent particle has a density of at least 1.5 g/ml, such as at least 1.8 g/ml, e.g. at least 2.0 g/ml, such as at least 2.5 g/ml, such as at least 2.6 g/ml, e.g. at least 3.0 g/ml, such as at least 3.5 g/ml, e.g. at

least 4.0 g/ml, such as at least 5 g/ml, e.g. at least 7 g/ml, such as at least 10 g/ml, e.g. at least 15 g/ml.

The density of an adsorbent particle is meant to describe the density of the adsorbent in its
5 fully solvated (e.g. hydrated) state as opposed to the density of a dried adsorbent.

The adsorbent particle used according to the invention must be at least partly permeable
to the bio-molecular substance to be isolated in order to ensure a significant binding
capacity in contrast to impermeable particles that can only bind the target molecule on its
10 surface resulting in relatively low binding capacity. The adsorbent particle may be of an
array of different structures, compositions and shapes.

Thus, the adsorbent particles may be constituted of a number of chemically derivatised
porous materials having the necessary density and binding capacity to operate at the given
15 flow rates per se. The particles are either of the conglomerate type, as described in WO
92/00799, having at least two non-porous cores surrounded by a porous material, or of
the pellicular type having a single non-porous core surrounded by a porous material.

In the present context the term "conglomerate type" relates to a particle of a particulate
20 material, which comprises beads of core material of different types and sizes, held
together by the polymeric base matrix, e.g. a core particle consisting of two or more high
density particles held together by surrounding agarose (polymeric base matrix).

In the present context the term "pellicular type" relates to a composite of particles,
25 wherein each particle consists of only one high density core material coated with a layer of
the porous polymeric base matrix, e.g. a high density stainless steel bead coated with
agarose.

Accordingly the term "at least one high density non-porous core" relates to either a
30 pellicular core, comprising a single high density non-porous particle or it relates to a
conglomerate core comprising more than one high density non-porous particle.

The adsorbent particle, as stated, comprises a high density non-porous core with a porous
material surrounding the core, and said porous material optionally comprising a ligand at
35 its outer surface.

In the present context the term "core" relates to the non-porous core particle or core
particles present inside the adsorbent particle. The core particle or core particles may be

incidental distributed within the porous material and is not limited to be located in the centre of the adsorbent particle.

The non-porous core constitutes typically of at most 50% of the total volume of the
5 adsorbent particle, such as at most 40%, preferably at most 30%.

Examples of suitable non-porous core materials are inorganic compounds, metals, heavy metals, elementary non-metals, metal oxides, non metal oxides, metal salts and metal alloys, etc. as long as the density criteria above are fulfilled. Examples of such core
10 materials are metal silicates metal borosilicates; ceramics including titanium diboride, titanium carbide, zirconium diboride, zirconium carbide, tungsten carbide, silicon carbide, aluminum nitride, silicon nitride, titanium nitride, yttrium oxide, silicon metal powder, and molybdenum disilicide; metal oxides and sulfides, including magnesium, aluminum, titanium, vanadium, chromium, zirconium, hafnium, manganese, iron, cobalt, nickel,
15 copper and silver oxide; non-metal oxides; metal salts, including barium sulfate; metallic elements, including tungsten, zirconium, titanium, hafnium, vanadium, chromium, manganese, iron, cobalt, nickel, indium, copper, silver, gold, palladium, platinum, ruthenium, osmium, rhodium and iridium, and alloys of metallic elements, such as alloys formed between said metallic elements, e.g. stainless steel; crystalline and amorphous
20 forms of carbon, including graphite, carbon black and charcoal. Preferred non-porous core materials are tungsten carbamide, tungsten, steel and titanium beads such as stainless steel beads.

The porous material is a polymeric base matrix used as a means for covering and keeping
25 multiple (or a single) core materials together and as a means for binding the adsorbing ligand.

The polymeric base matrix may be sought among certain types of natural or synthetic organic polymers, typically selected from i) natural and synthetic polysaccharides and
30 other carbohydrate based polymers, including agar, alginate, carrageenan, guar gum, gum arabic, gum ghatti, gum tragacanth, karaya gum, locust bean gum, xanthan gum, agaroses, celluloses, pectins, mucins, dextrans, starches, heparins, chitosans, hydroxy starches, hydroxypropyl starches, carboxymethyl starches, hydroxyethyl celluloses, hydroxypropyl celluloses, and carboxymethyl celluloses; ii) synthetic organic polymers and
35 monomers resulting in polymers, including acrylic polymers, polyamides, polyimides, polyesters, polyethers, polymeric vinyl compounds, polyalkenes, and substituted derivatives thereof, as well as copolymers comprising more than one such polymer functionally, and substituted derivatives thereof; and iii) mixture thereof.

A preferred group of polymeric base matrices are polysaccharides such as agarose.

From a productivity point of view it is important that the adsorbent is able to bind a high amount of the bio-molecule per volume of the adsorbent.

5

The preferred shape of a single adsorbent particle is substantially spherical. The overall shape of the particles is, however, normally not extremely critical, thus, the particles can have other types of rounded shapes, e.g. ellipsoid, droplet and bean forms. However, for certain applications (e.g. when the particles are used in a fluidised bed set-up), it is

10 preferred that at least 95% of the particles are substantially spherical.

Preparation of the particulate material according to the invention may be performed by various methods known per se (e.g. by conventional processes known for the person skilled in the art, see e.g. EP 0 538 350 B1 or WO 97/17132. For example, by block

15 polymerisation of monomers; suspension polymerisation of monomers; block or suspension gelation of gel-forming materials, e.g. by heating and cooling (e.g. of agarose) or by addition of gelation "catalysts" (e.g. adding a suitable metal ion to alginates or carrageenans); block or suspension cross-linking of suitable soluble materials (e.g. cross linking of dextrans, celluloses, or starches or gelatines, or other organic polymers with e.g. 20 epichlorohydrin or divinyl sulphone); formation of silica polymers by acidification of silica solutions (e.g. block or suspension solutions); mixed procedures e.g. polymerisation and gelation; spraying procedures; and fluid bed coating of density controlling particles; cooling emulsions of density controlling particles suspended in polymeric base matrices in heated oil solvents; or by suspending density controlling particles and active substance in 25 a suitable monomer or copolymer solution followed by polymerisation.

In a particularly suitable embodiment generally applicable for the preparation of the particulate material according to the invention, a particulate material comprising agarose as the polymeric base matrix and steel beads as the core material is obtained by heating a

30 mixture of agarose in water (to about 95°C), adding the steel beads to the mixture and transferring the mixture to a hot oil (e.g. vegetable oils), emulsifying the mixture by vigorous stirring (optionally by adding a conventional emulsifier) and cooling the mixture. It will be appreciated by the person skilled in the art that the particle size (i.e. the amount of polymeric base matrix (here: agarose) which is incorporated in each particle can be 35 adjusted by varying the speed of the mixer and the cooling process. Typically, following the primary production of a particle preparation the particle size distribution may be further defined by sieving and/or fluid bed elutriation.

The porous matrix, such as polymer agarose, is typically chemically derivatised with a low molecular weight compound referred to herein as the ligand and the adsorbent comprises a ligand with affinity to proteins. The ligand constitutes the adsorbing functionality of the adsorbent media or the polymeric backbone of the adsorbent particle has a binding functionality incorporated per se. Well-known ligand chemistries such as cation exchangers, e.g. sulphonic acid, have been proven to be efficient tools for purification of whey proteins such as lactoferrin and lactoperoxidase. These proteins are positively charged, even at neutral pH, and selective interaction with a cation exchanger can be obtained. Other proteins require more sophisticated binding interaction with the ligand in order to obtain a selective adsorption.

Such affinity ligands, like the chargeable moieties, may be linked to the base matrix by methods known to the person skilled in the art, e.g. as described in "Immobilized Affinity Ligand Techniques" by Hermanson et al., Academic Press, Inc., San Diego, 1992. In cases where the polymeric base matrix do not have the properties to function as an active substance, the polymeric base matrix (or matrices where a mixture of polymers are used) may be derivatised to function as an active substances in the procedures of activation or derivatisation. Thus, materials comprising hydroxyl, amino, amide, carboxyl or thiol groups may be activated or derivatised using various activating chemicals, e.g. chemicals such as cyanogen bromide, divinyl sulfone, epichlorohydrin, bisepoxyranes, dibromopropanol, glutaric dialdehyde, carbodilimides, anhydrides, hydrazines, periodates, benzoquinones, triazines, tosylates, tresylates, and diazonium ions.

Specifically preferred methods for chemical derivatization and specific ligands applicable according to this invention is described in WO 98/08603.

In order to ensure an optimal adsorption strength and productivity of the adsorbent it has been found that the ligand concentration on the adsorbent is very significant. Thus, in a suitable embodiment, the adsorbent carries ligands for adsorption of the biomolecular substances in a concentration of at least 20 nM, such as at least 30 mM or at least 40 mM, preferably at least 50 mM and most preferably at least 60 mM.

A subset of adsorbents may be characterised in terms of their binding capacity to bovine serum albumin (BSA). This subset of adsorbents are typically those comprising a ligand selected from the group consisting of i) ligands comprising aromatic or heteroaromatic groups (radicals) of the following types as functional groups: benzoic acids such as 2-aminobenzoic acids, 3-aminobenzoic acids, 4-aminobenzoic acids, 2-mercaptobenzoic acids, 4-amino-2-chlorobenzoic acid, 2-amino-5-chlorobenzoic acid, 2-amino-4-chlorobenzoic acid, 4-aminosalicylic acids, 5-aminosalicylic acids, 3,4-diaminobenzoic

- acids, 3,5-diaminobenzoic acid, 5-aminoisophthalic acid, 4-aminophthalic acid; cinnamic acids such as hydroxy-cinnamic acids; nicotinic acids such as 2-mercaptopicotinic acids; naphthoic acids such as 2-hydroxy-1-naphthoic acid; quinolines such as 2-mercaptopquinoline; tetrazolacetic acids such as 5-mercpto-1-tetrazolacetic acid;
- 5 thiadiazols such as 2-mercpto-5-methyl-1,3,4-thiadiazol; benzimidazols such as 2-amino-benzimidazol, 2-mercaptopbenzimidazol, and 2-mercpto-5-nitrobenzimidazol; benzothiazols such as 2-aminobenzothiazol, 2-amino-6-nitrobenzothiazol, 2-mercaptopbenzothiazol and 2-mercpto-6-ethoxybenzothiazol; benzoxazols such as 2-mercaptopbenzoxazol; thiophenols such as thiophenol and 2-aminothiophenol; 2-(4-
- 10 aminophenylthio)acetic acid; aromatic or heteroaromatic sulfonic acids and phosphonic acids, such as 1-amino-2-naphthol-4-sulfonic acid and phenols such as 2-amino-4-nitrophenol. It should be noted that the case where M is agarose, SP1 is derived from vinyl sulfone, and L is 4-aminobenzoic acid is specifically disclaimed in relation to the solid phase matrices according to the invention, cf. WO 92/16292, most preferably amino-
- 15 benzolic acids like 2-amino-benzoic acid, 2-mercpto-benzolic acid, 3-aminobenzolic acid, 4-aminobenzoic acid, 4-amino-2-chlorobenzolic acid, 2-amino-5-chlorobenzoic acid, 2-amino-4-chlorobenzolic acid, 4-aminosalicylic acids, 5-aminosalicylic acids, 3,4-diaminobenzolic acids, 3,5-diaminobenzoic acid, 5-5-aminoisophthalic acid, 4-aminophthalic acid; ii) ligands comprising 2-hydroxy-cinnamic acids, 3-hydroxy-cinnamic acid and 4-hydroxy-cinnamic
- 20 acid iii) ligands comprising a carboxylic acid and an amino group as substituents such as 2-amino-nicotinic acid, 2-mercpto-nicotinic acid, 6-amino-nicotinic acid and 2-amino-4-hydroxypyrimidine-carboxylic acid iv) ligand comprising radicals derived from a benzene ring fused with a heteroaromatic ring system, e.g. a ligand selected from benzimidazoles such as 2-mercpto-benzimidazol and 2-mercpto-5-nitro-benzimidazol; benzothiazols
- 25 such as 2-amino-6-nitrobenzothiazol, 2-mercaptopbenzothiazol and 2-mercpto-6-ethoxybenzothiazol; benzoxazols such as 2-mercaptopbenzoxazol; and v) ligands chosen from the group of thiophenols such as thiophenol and 2-aminothiophenol.

- Within the embodiment wherein the ligand is selected from group i)-v), the adsorbents
- 30 typically have a dynamic binding capacity of at least 10 g of biomolecular substance per litre, more preferably at least 20 g per litre, still more preferable at least 30 g per litre when tested according to the process conditions used in the relevant application. The binding capacity of the adsorbent may be determined in terms of its binding capacity to bovine serum albumin (BSA). The binding capacity is typically such that at least 10g/L of
- 35 BSA binds according to test Method A.

Method A is a method used for determination of the bovine albumin binding capacity of selected adsorbents consisting of the following process:

Bovine serum albumin solution pH 4.0 (BSA pH 4.0): Purified bovine serum albumin (A 7906, Sigma, USA) is dissolved to a final concentration of 2 mg/ml in 20 mM sodium citrate pH 4.0. Adsorbents are washed with 50 volumes of 20 mM sodium citrate pH 4.0 and drained on a suction filter.

5

A sample of 1.0 ml suction drained adsorbent is placed in a 50 ml test tube followed by the addition of 30 ml of BSA, pH 4.0.

The test tube is then closed with a stopper and the suspension incubated on a roller mixer
10 for 2 hours at room temperature (20-25 °C). The test tube is then centrifuged for 5 min. at 2000 RPM in order to sediment the adsorbent completely. The supernatant is then isolated from the adsorbent by pipetting into a separate test tube, avoiding the carry-over of any adsorbent particles and filtered through a small non-adsorbing 0.2 µm filtre (Millipore, USA). Following this a determination of the concentration of non-bound BSA in the
15 supernatant is performed by measuring the optical density (OD) at 280 nm on a spectrophotometer.

The amount of BSA bound to the adsorbent is then calculated according to the following formula:

20 *mg BSA bound per ml suction drained adsorbent =*
(1-(OD of test supernatant/OD of BSA starting solution)) x 60 mg BSA/ml adsorbent.

Washing

In a preferred embodiment the washing liquid is water e.g. tap water, demineralised
25 water, water produced by reverse osmosis or distilled water.

In a preferred embodiment of the present invention the flow rate used for the washing steps involved is selected from the ranges outlined previously for conventional methodologies. These are generally much lower than the linear flow rate used when
30 loading the bio-molecule containing fluid onto the column.

Elution

The one or more bio-molecule(s) of interest is/are released from the adsorbent using an eluent such as a buffer or any other solution capable of changing for example the pH
35 within the column and which produces a generally clear and concentrated solution of the one or more bio-molecule(s).

Appropriate eluents depend on the type of adsorbent and the elution may be performed by any method conventionally described and known in the art.

In some embodiments of the invention, wherein the bio-molecule is a protein, the elution of the adsorbed protein is performed with a solution, typically selected from the group consisting of dilute base, dilute acid, and water. In the embodiment wherein the eluting or 5 washing step is performed with such a solution, the solution is dilute so as to minimise the amount of salt and other unwanted substances present in the eluted product.

Thus, in a preferred embodiment the dilute acid or base used for elution of the bio- molecule has a salt concentration of less than 50 mM, preferably less than 30 mM, even 10 more preferable less than 20 mM. The determination of the salt concentration is performed directly on the eluate fraction containing the protein or proteins to be isolated without additional dilution of the eluate fraction. Common, low cost and non-toxic acids and bases are applicable. Specifically preferred are the bases sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide (Ca(OH)₂), ammonium hydroxide (NH₄OH).
15

In a preferred embodiment of the present invention the flow rate used for the elution step or steps involved is selected from the ranges outlined previously for applying the protein-containing mixture to the adsorbent column.

20 EXAMPLES

Example 1.

Isolation of Lactoferrin (LF) from skimmed milk using expanded bed adsorption chromatography at 10°C versus 50°C:

Non-pasteurised skim milk with pH 6.6 was obtained from a local dairy company.
25

Adsorbent

FastLine SP, product number 900-1600 UpFront Chromatography.

The adsorbent is based on agarose with tungsten carbide particles incorporated, density of approximately 2.9 g/ml, particle size in the range of 40-200 µm with a mean particle size

30 of 80µm, strong cation exchanger comprising sulfonic groups.

Pre-treatment of the non-pasteurised skimmed milk

For running the experiment at 10°C the skimmed milk was equilibrated to a temperature of 10°C and kept at 10°C during the experiment.
35

For running the experiment at 50°C the skimmed milk was pumped through a heat exchanger to reach 50°C before it was loaded onto the column. No pH adjustment was performed.

5 Process parameters

The experiment was performed in a FastLine[®]300 expanded bed column ($\varnothing=30$ cm) product number 7300-0000, UpFront Chromatography.

- 10 The column was packed with a sedimented bed height of 15 cm of adsorbent (10.6 l) and equilibrated with demineralised water at 10°C and 50°C, respectively.

3180 l of the skimmed milk was loaded onto the column with a linear flow rate of 1.500 cm/hr.

- 15 The column was washed with aqueous buffer pH 6.5 containing 25 mM of sodium citrate and 0.15 M of sodium chloride. Lactoferrin was then eluted using a solution of 20mM sodium hydroxide.

Determination of Lactoferrin

- 20 The concentration of lactoferrin in the eluate was determined by Single Radial Immunodiffusion (RID) using goat anti-bovine lactoferrin from Bethyl Laboratories inc. (1 μ l per cm^2) as described in Scand. J. Immunol. Vol. 17, Suppl. 10, 41-56, 1983. The concentration was calculated from a standard curve produced with well known concentrations of lactoferrin from Sigma (cat. no. L 9507).

25

Results

The table below shows the volumes of skimmed milk and buffers loaded onto each column:

| Fraction | Process running at 10°C | Process running at 50°C |
|---------------------------------------|----------------------------|----------------------------|
| Volume of skimmed milk loaded, litres | 3180 | 3180 |
| Volume of washing solutions, litres | 174 | 210 |
| Elution of lactoferrin, litres | 105 | 114 |
| Total volume processed, litres | 3459 | 3504 |
| Process time, hr | 3.26 | 3.31 |

The actual flow rate through the columns is 100 l/hr/l of adsorbent.

The table below shows the results from the two experiments. (LF = Lactoferrin)

| Temperature °C | g LF in eluate | Adsorbent capacity g LF/l adsorbent | Expansion of adsorbent during load of skimmed milk | Productivity g LF/l adsorbent/hr |
|-------------------|-------------------|--|---|--|
| 10 | 244 | 23 | 10 times | 7.1 |
| 50 | 461 | 44 | 4 times | 13.2 |

5

The results show that the productivity, as presented by the amount of Lactoferrin isolated per litre adsorbent in one hour, is higher when the process is operated at 50°C than when operated at 10°C.

10 Example 2

Isolation of lactoferrin from non-pasteurised skimmed milk using expanded bed chromatography at linear flow rates of 1,500, 2,100 or 3,000 cm/hr at 50°C.

All conditions except for the flow rates were the same as described in example 1.

15

Results

The table below shows the volumes of skimmed milk and buffers loaded onto each column:

| Fraction | Process running at 1500 cm/hr | Process running at 2100 cm/hr | Process running at 3000 cm/hr |
|--|----------------------------------|----------------------------------|----------------------------------|
| Volume of skimmed milk loaded, litres | 3180 | 3180 | 3180 |
| Volume of washing solutions, litres | 210 | 232 | 302 |
| Elution of lactoferrin, litres | 114 | 115 | 192 |
| Total volume processed, litres | 3504 | 3527 | 3674 |
| Process time, hr | 3.3 | 2.4 | 1.7 |

20

The table below shows the results from the three experiments. (LF = Lactoferrin)

| Flow rate cm/hr | Volume loaded l/hr/l adsorbent | g LF in eluate | Adsorbent capacity g LF/l adsorbent | Expansion of adsorbent during load of skimmed milk | Productivity g LF/l adsorbent/hr |
|--------------------|---|-------------------|--|---|--|
| 1,500 | 100 | 466 | 44 | 3.9 times | 13.3 |
| 2,100 | 140 | 456 | 43 | 4.4 times | 17.9 |
| 3,000 | 200 | 445 | 42 | 8 times | 24.7 |

The results show that when operating the expanded bed column at 50°C and the linear flow rate increase from 1500 to 3000 cm/hr, the process productivity and volume loaded

- 5 per hour per litre of adsorbent increases significantly.

Example 3

Isolation of lactoferrin from sweet whey using expanded bed adsorption chromatography at 16°C versus 50°C.

10

Process parameters

The experiment was performed in a FastLine®300 expanded bed column ($\varnothing=30$ cm) product number 7300-0000, UpFront Chromatography.

- 15 A column was packed with 15 cm of adsorbent (10.6 l) and equilibrated with demineralised water at 16°C or 50°C.

3180 l of sweet whey was loaded onto the column with a linear flow rate of 900 and 1,500 cm/hr, respectively.

20

The column was washed with aqueous buffer pH 6.5 containing 25 mM of sodium citrate and 0.30 M of sodium chloride. Lactoferrin was then eluted using a solution of 20mM sodium hydroxide.

25

30

Results

The table below shows the volumes of sweet whey and buffers loaded onto each column:

| Fraction | Process at flow rate 1500 cm/hr, 16°C | Process at flow rate 2100 cm/hr, 50°C |
|---------------------------------------|--|--|
| Volume of skimmed milk loaded, litres | 3180 | 3180 |
| Volume of washing solutions, litres | 75 | 180 |
| Elution of lactoferrin, litres | 73 | 150 |
| Total volume processed, litres | 3328 | 3510 |
| Process time, hr | 3.1 | 2.4 |

5

The table below shows the results from the two experiments. (LF = Lactoferrin)

| Flow rate cm/hr | Volume loaded l hr/l adsorbent | T °C | g LF in eluate | Adsorbent capacity g LF/l adsorbent | Expansion of adsorbent | Productivity g LF/l adsorbent/hr |
|-----------------|--------------------------------|------|----------------|-------------------------------------|------------------------|----------------------------------|
| 1,500 | 100 | 16 | 167 | 15.8 | 4 times | 5.1 |
| 2,100 | 140 | 50 | 158 | 14.9 | 3.3 times | 6.2 |

The results indicate that it is possible to increase the flow rate from 1,500 to 2,100 cm/hr if the temperature is increased from 16 to 50°C and still obtain a high productivity.

10

Example 4

Isolation of whey proteins from sweet whey using expanded bed adsorption.
at 1,500 cm/hr.

- 15 Sweet whey was obtained from a local dairy company pH 6.3.

Adsorbent

FastLine PRO, UpFront Chromatography.

The adsorbent is based on agarose with tungsten carbide particles incorporated, density of approximately 2.9 g/ml, particle size in the range of 40-200 µm with a mean particle size of 80µm. The adsorbent comprises a mixed mode ligand comprising an aromatic ring structure with a carboxylic acid substituent. The adsorbent binds molecules in the pH 5 range of 3 to 6. The molecules are released by increasing the pH in the elution buffer to above 7.

Pre-treatment of sweet whey

- For running the experiment at 50 °C the sweet whey was pumped through a heat exchanger to reach 50 °C before it was loaded onto the column.

pH was adjusted to 4.7 with 1 M hydrochloric acid.

Process parameters

- 15 The experiment was performed in a FastLine™300 expanded bed column ($\varnothing=30$ cm) product number 7300-0000, UpFront Chromatography.

The column was packed with 15 cm of adsorbent (10.6 l) and equilibrated with demineralised water at 50°C.

- 20 160 l of sweet whey was loaded onto the column with a linear flow rate of 1,500 cm/hr. The volume flow through from the column was collected in three fractions.

- Non-bound material was washed out with demineralised water (290 l). The bound proteins were eluted in two steps.

Step 1: 50 mM adipic acid, 0.1 mg/ml SDS (sodium dodecylsulfate) pH 5.3 (275 l).

Step 2: 20 mM NaOH (117 l).

Results

- 30 Each fraction from the experiment was tested with SDS-PAGE to evaluate the content and nature of proteins.

SDS PAGE

For SDS PAGE, Invitrogen SDS Page 4-20 % Tris-Glycine gel (cat no. EC6025) was used.

- 35 Sample preparation: 25µl sample and 25µl sample buffer Tris-Glycine Invitrogen (cat no. LC2676) was mixed and boiled for 5 minutes in a water bath. The running buffer 0.024 M Tris (Sigma T1378), 0.19 M Glycine (Merck 5001901000), 0.1 % SDS (Sodium dodecyl sulphate, JT Baker 2811) pH 8.6 was added.

20 µl sample was applied in each analysis slot and the power was adjusted to give a current of 40 mA. When the blue line from the sample buffer reached one cm from the bottom of the gel the power was turned off and the gel was stained overnight in

- 5 Invitrogen's Colloidal Blue Staining Kit (cat. no. LC 6025) on a shaking table. The next day the gel was transferred into water and de-stained in water for 2 hours.

Figure 1 (SDS-PAGE) shows that protein content is highly reduced in the three flow-through fractions from the column, the major part of the immunoglobulin G, bovine serum albumin, β -lactoglobulin and α -lactalbumin is bound to the adsorbent (lane 2-4)
10 In elution step 1: 50 mM adipic acid, 0.1 mg/ml SDS (sodium dodecylsulfate) pH 5.3, all the bound, β -lactoglobulin is recovered (lane 6, figure 1).
In elution step 2: 20 mM all the bound the immunoglobulin G, bovine serum albumin and α -lactalbumin is recovered (lane 7, figure 1).

21 MRS. 2003

CLAIMS

1. A process for isolation of one or more bio-molecule(s) from a bio-molecule containing fluid comprising the steps of:

- 5 a) optionally adjusting the pH of the bio-molecule containing fluid;
- b) optionally bringing the bio-molecule containing fluid to a temperature of between 45°C to 80°C;
- 10 c) applying a volume of said bio-molecule containing fluid to a chromatographic column comprising an adsorbent, said chromatographic column is operated with a temperature of between 45°C to 80°C and a linear flow rate of at least 1.500 cm/hour;
- 15 d) optionally washing the column;
- e) eluting at least one bio-molecule from the adsorbent.

2. The process according to claim 1, wherein the chromatographic column is a large-scale chromatographic column comprising at least 10 l of sedimented adsorbent.

3. The process according to any one of claims 1 or 2, wherein the chromatographic column is a large-scale chromatographic column comprising from about 50 to 100 l of sedimented adsorbent, preferably from about 100 to 1000 l of adsorbent, more preferably from about 25 200 to 900 l of adsorbent, most preferably from about 300 to 800 l of adsorbent.

4. The process according to any of the preceding claims, wherein the chromatographic column has a diameter of at least 10 cm, preferably of at least 20 cm, more preferably in the range of from about 50 cm to 200 cm, such as 100 to 150 cm.

30 5. The process according to any of the preceding claims, wherein the one or more bio-molecule(s) has a molecular weight of at least 1000 Daltons, preferably of at least 1500 Daltons, more preferably of at least 2000 Daltons.

- 35 6. The process according to any of the preceding claims, wherein the one or more bio-molecule(s) is/are selected from the group consisting of peptides, proteins, lipids, lipoproteins, polysaccharides, cell constituents, cells and combinations thereof.

7. The process according to claim 6, wherein said proteins is selected from the group consisting of lactoferrin, β -lactoglobulin, α -lactalbumin and lactoperoxidase.

8. The process according to claim 6, wherein said polysaccharide is starch.

5

9. The process according to any of the preceding claims, wherein the bio-molecule containing fluid is selected from the group consisting of body fluids, fermentation fluids, waste water, process water, plant extracts, tissue extracts, synthesis mixtures and fluids derived therefrom.

10. The process according to claim 8, wherein the body fluid is selected from the group consisting of milk, plasma, urine, egg white and fluids derived therefrom.

15 11. The process according to any of the preceding claims, wherein the adsorbent consists of adsorbent particles wherein 50% of the number of particles has a particle size of at most 200 μm , such as at most 175, 150, 120, 100 or 80 μm .

12. The process according to any of the preceding claims, wherein the adsorbent consists
20 of adsorbent particles with a mean particle size of at most 200 μm , such as at most 150 μm , particularly at most 120 μm , more particularly at most 100 μm , even more particularly at most 90 μm , even more particularly at most 80 μm , even more particularly at most 70 μm . Typically the adsorbent particle has a mean particle size in the range of 40-150 μm , such as 40-120 μm , e.g. 40-100, such as 40-75, e.g. 40-50 μm .

25

13. The process according to any of the preceding claims, wherein the linear flow-rate is from about 1.500 to 12.000 cm/hr, preferably from about 1.800 to 10.000 cm/hr, such as about 3000 cm/hr.

30 14. The process according to any of the preceding claims, wherein the volume applied is from about 2-3500 l/min.

15. The process according to any of the preceding claims, wherein the volume applied per litre of adsorbent in one hour is at least 50 l, preferably at least 100 l, more preferably at 35 least 150 l/min such as at least 200 l/min.

16. The process according to any of the preceding claims, wherein the chromatographic column is an expanded bed adsorption column.

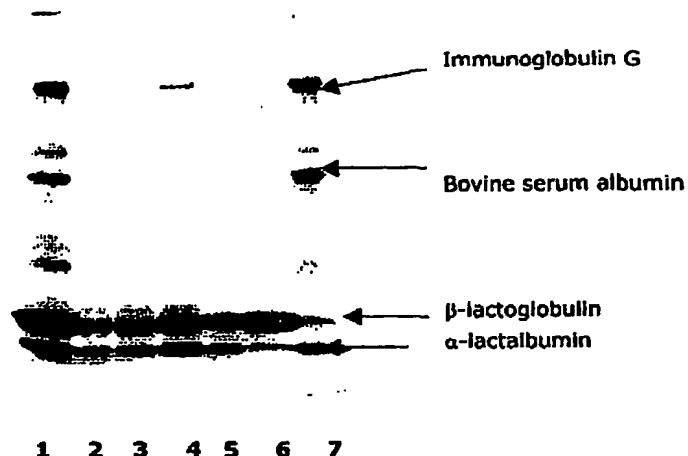
27

17. The process according to any of the preceding claims, wherein the chromatographic column is a packed bed adsorption column.
18. The process according to claim 17, wherein the pressure measured over the chromatographic column is at most 10 bar, such as at most 9, 8, 7, 6 or 5 bar, preferably of at most 4 bar, most preferably of at most 3 bar such as of at most 2.5 bar.

28

Modtaget PVS
21.MRS. 2003

Figure 1.



**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.